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Rapid assessment of *Oenococcus oeni* activity by measuring intracellular pH and membrane potential by flow cytometry, and its application to the more effective control of malolactic fermentation ☆

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ABSTRACT

The aim of this study is to highlight the changes in the physiological cellular state of *Oenococcus oeni* during malolactic fermentation (MLF), and to use its cellular parameters to improve existing knowledge of *O. oeni* behaviour and to more effectively control the performance of the bacteria during MLF in wine.
To do this, measurements of intracellular pH, transmembrane potential and vitality were performed using flow cytometry with different fluorescent probes: CFDA-SE and CDCF, DiBAC and CFDA, respectively. The kinetics of the cellular changes in these parameters were determined during MLF in FT80 synthetic medium and in white wine, as were the kinetics of malic acid consumption.
pH_{in} measurement throughout the entire growth shows that the pH was equal to the pH of the culture medium during the early stage, increased to pH 6 in the exponential phase, and then decreased to equilibrate with the pH of the medium in the late stationary phase. Membrane potential increased in early MLF and then decreased. The decrease in pH_{in} and membrane potential occurred when all of the malic acid was consumed. Finally, we showed that the higher the ΔpH ($pH_{in} - pH_{ex}$) in *O. oeni* cells was, the shorter the lag phase of the MLF was. To better manage the initiation of MLF in wines, the physiological state of *O. oeni* cells must be taken into account. These results allow us to understand the sometimes random initiation of MLF in wines inoculated with *O. oeni* and to suggest ways to improve this control.

1. Introduction

Malolactic fermentation (MLF) takes place after alcoholic fermentation and consists of the decarboxylation of malic acid into lactic acid and CO_2. As a consequence, the organoleptic quality and the microbiological stability of wine are improved. MLF is mainly driven by the lactic acid bacterium *Oenococcus oeni*, which is able to decarboxylate malic acid under unfavourable conditions such as low pH (Drici-Cachon et al., 1996), high ethanol concentration and the presence of other compounds from yeast metabolism (Lonvaud-Funel, 1999). In practice, it is difficult to control MLF in wines because the cell physiology of *O. oeni* is poorly known. One criterion of growth physiology is the culturability measured using the plate count technique. However, in the case of *O. oeni*, the plate count technique requires a very long incubation time of about seven to ten days, which is not compatible with a real-time control of MLF. Moreover, viable and culturable cells can be weakly active or very active. This technique is not sufficient to assess the malolactic activity and effectiveness of the bacteria. Activity can be measured by kinetic parameters such as lag phase, specific growth rate, specific substrate degradation rate or end product formation rate, but the assessment of all of these parameters also requires a long time (Rault et al., 2008).

Bacteria have developed several mechanisms that allow them to survive and grow in an acidic environment. For example, some lactic acid bacteria maintain the transmembrane pH gradient constant by decreasing their intracellular pH (pH_{in}) when extracellular pH (pH_{ex}) decreases (Siegumfeldt et al., 2000). The mechanisms by which lactic acid bacteria regulate their pH_{in} are based on the structure and the function of the plasma membrane (Garbay and Lonvaud-Funel, 1994). In *O. oeni*, the conversion of L-malic acid to L-lactic acid is not energetic, and the production of ATP is obtained by a chemiosmotic mechanism that involves the two components of the proton motive force, ΔpH and Δψ, which is sufficient to drive ATP synthesis by membrane ATPase (Cox and Henick-Kling, 1989, 1995; Olsen et al., 1991; Salema et al., 1994). In *O. oeni*, the ATPase complex is of the $(F_1F_0)H^+$-ATPase type (Salema et al., 1996b) and can function reversibly when coupled with a proton pump via the synthesis of ATP. In wine (acidic medium), this ATPase activity is very important to the survival of *O. oeni*. The role of ATPase in relation to acid tolerance has been demonstrated with ATPase-deficient mutants (Tourdot-Maréchal et al., 1999). Since all the ATPase-deficient mutants lack malolactic activity, a link has been suggested between ATPase and malolactic activity in *O. oeni* (Galland

☆ This work is dedicated to the memory of Monique Charpentier.

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et al., 2003), and, moreover, it has been observed that ATPase activity was maximal in the exponential growth phase of *O. oeni* (Carrete et al., 2002). It has been demonstrated (Salema et al., 1996b) that MLF generates both a transmembrane pH gradient and an electrical potential gradient $\Delta\Psi$ in *O. oeni*, and that the latter controls the rate of fermentation. It has also been shown that malate influx leads to the increase of the negative charge in the cell, subsequently improving membrane polarisation (Loubiere et al., 1992; Salema et al., 1994, 1996a) and decreasing the $\Delta\Psi$ at the end of MLF when malate is exhausted. Cox and Henick-Kling (1995) observed a rise in pH_{in} due to the consumption of protons by the malolactic enzyme, and some authors hypothesized that the lactic acid and carbon dioxide were co-extruded in symport with protons (Poolman et al., 1991; Salema et al., 1994). The generated ΔpH was the major component of the proton motive force responsible for the ATP generation observed (Cox and Henick-Kling, 1995). Metabolism is thus closely linked to the cellular parameters ΔpH and $\Delta\Psi$, but most of the studies were performed on cells in the stationary phase or in vesicles (Salema et al., 1994), or on cells in the middle of the exponential phase (Augagneur et al., 2007), and always on only one point of the growth curve. Consequently, there is a lack of information about the kinetics of these phenomena. We think that the kinetics of changes in the pH_{in} and the membrane potential $\Delta\Psi$ during MLF could make it possible to improve existing knowledge about *O. oeni* behaviour and to better control the performance of the bacteria in MLF.

Flow cytometry (FCM) is a rapid method for cell-by-cell analysis. It can be applied in combination with several fluorescent probes. It is used to count viable bacteria (Jepras et al., 1995) and yeasts (Bouix et al., 1999). Esterase substrates such as fluorescein diacetate (FDA) and carboxyfluorescein diacetate (cFDA) are widely used for viability assessment (Breeuwer and Abee, 2000b; Bunthof et al., 2001; Ben Amor et al., 2002). However, due to the change in pH_{in} during growth, we have previously shown that it is preferable to use BacLight™ staining rather than cFDA staining to enumerate *O. oeni* (Bouix and Ghorbal, 2013).

cFDA is a non-fluorescent precursor that is taken up by the cell and converted by non-specific esterase to a fluorescent compound, cF. cF can be actively extruded from the cells (Breeuwer et al., 1994), and this extrusion can be used as a tool to assess the vitality of yeasts (Bouix and Leveau, 2001), *Lactobacillus* (Rault et al., 2008) and *O. oeni* (Da Silveira and Abee, 2009). FCM is also used to characterise cellular physiological parameters such as membrane potential and pH_{in}. Bis-(1,3-bibutylbarbituric acid) trimethine oxonol (DiBAC4(3)) is the most commonly used dye for bacteria among the membrane potential-sensitive probes. Oxonols are anionic probes that enter depolarised cells and bind to lipid-rich compounds, resulting in green fluorescence (Mason et al., 1995). The more the cells are depolarised, the brighter the green fluorescence will be (Gabier et al., 2005). The main dyes used to assess pH_{in} are 5,6 carboxyfluorescein diacetate succimidyl ester (cFDA-SE) for the 5 to 7 range of pH_{in} (Breeuwer et al., 1996), and 5–6 carboxy 2′7′-dichlorofluorescein (CDCF) for the lower pH_{in} range (Nedergaard et al., 1990). By using different probes and FCM, it is possible to assess the viability, the vitality, the membrane potential and the pH_{in} of microbial cells in a short time.

The aim of this study was, first, to highlight the change in the components of the proton motive force, energetic state and vitality of *O. oeni* cells during MLF, using FCM. We then determined the best physiological cellular state for inoculation of bacteria in wine in order to better control MLF.

2. Materials and methods

2.1. Strain and cultures

The MC1 strain of *O. oeni* was isolated from wine by Moet et Chandon, and was stored at −80 °C. Unlike most of the *O. oeni* strains, MC1 does not metabolise citric acid. All the cultures were performed in a flask, in FT80 synthetic medium (glucose: 5 g, fructose: 5 g, L-malic acid: 5 g, yeast extract: 5 g, beef peptone: 2.5 g, tryptone: 2.5 g, NaH_2PO_4, $2H_20$: 0.5 g, $MgCl_2$: 0.05 g, $MnSO_4,H_20$: 0.01 g, KCl: 0.15 g, $CaCl_2$: 0.13 g, Tween 80: 1 mL, qsp water 1 L) (Cavin et al., 1989), adjusted to pH 3.2 or pH 5 before sterilisation at 120 °C, 20 min, and incubated at 30 °C. Some cultures were performed in white wine (grape variety: Pinot Meunier, 11% ethanol, SO_2: 40 mg/L, pH 3.2) (provided by Moet et Chandon), supplemented with L-malic acid (final concentration: 5 $g.L^{-1}$), filtered on a 0.45-μM membrane to eliminate other bacteria, and incubated at 20 °C after inoculation with MC1. Plate count was performed in FT80 agar (FT80 broth, 15 $g.L^{-1}$ agar). All the pre-cultures were performed in the FT80 broth at pH 5 for 96 h. The culture in synthetic medium and in wine was inoculated at a concentration corresponding to approximately 10^6 $cells.mL^{-1}$ (optical density of 0.025 at 600 nM).

Bacterial growth was monitored using two methods. The enumeration of culturable cells was performed by plating appropriate serial dilutions on FT80 agar. The count was obtained as the number of colony-forming units after incubation for 15 days at 30 °C. The enumeration of viable and dead cells was performed by flow cytometry after fluorescence staining with the Live-Dead BacLight™ kit, as described below. All the measurements were performed once a day during MLF. Each data point was the mean of three independent cultures.

2.2. Probes

Chemchrome V8 containing carboxyfluorescein diacetate (cFDA) (AES-Chemunex, Ivry sur Seine, France), 5,6 carboxy 2′,7′ dichlorofluorescein diacetate (CDCF), 5,6 carboxyfluorescein diacetate succimidyl ester (cFDA SE), bis-(1,3-dibutylbarbituric acid) ($DiBAC_4(3)$), and the Live-Dead BacLight™ Viability kit were obtained from Molecular Probes (Life Technologies, St. Aubin, France).

2.3. Live-Dead BacLight™ staining

One millilitre of culture containing approximately 10^6 cells was centrifuged in an MSE Micro Centaur SANYO centrifuge (13,000 *g*, 1 min) and the pellet was suspended in 1 mL of McIlvaine's buffer at pH 7.3 (citric acid: 0.1 M; disodium hydrogenophosphate: 0.2 M), and incubated for 15 min at room temperature in the dark in the presence of 1.5 μL of Syto9 and 1.5 μL of PI (solutions ready to use in the kit) (Bouix and Ghorbal, 2013).

2.4. CFDA, CDCF and CFDA SE staining

One millilitre of culture containing approximately 10^6 cells was centrifuged in an MSE Micro Centaur SANYO centrifuge (13,000 *g*, 1 min). The pellet was resuspended in 1 mL of McIlvaine's buffer at pH 7.3, or in phosphate buffer (orthophosphoric acid: 0.1 M; disodium hydrogenophosphate: 0.2 M), and incubated for 10 min at 40 °C in the presence of 10 μL of Chemchrome V8, 10 μL of CDCF (188.9 mM in acetone) or 5 μL of cFDA SE (8.96 mM in acetone).

2.5. Viability measurement

The BacLight™ double-stained sample was centrifuged (13,000 *g*, 1 min) and the pellet was suspended in McIlvaine's buffer at pH 3.2 before FCM analysis.

2.6. Vitality measurement

Based on the energy-dependent extrusion of cF from the cell after conversion of cFDA (Breeuwer et al., 1994), we developed a vitality descriptor on the same principle as for yeast (Bouix and Leveau, 2001) or *Lactobacillus* (Rault et al., 2008). Two millilitres of the ChemChrome-stained sample were centrifuged (13,000 *g*, 90 s), and the pellet was resuspended in 2 mL of McIlvaine's buffer at pH 3.2. One millilitre was

immediately analysed by FCM to measure the fluorescence intensity FI_0. One millilitre was incubated for 15 min at 40 °C, centrifuged, and the pellet was resuspended in McIlvaine's buffer (pH 3.2) before FCM analysis. The fluorescence intensity FI_{15} was measured. The excretion percentage was calculated as follows: $\Delta FI_{15} = 100(FI_0 - FI_{15}) / FI_0$. The more the cells were active, the greater the excretion and the descriptor ΔFI_{15} were.

2.7. Intracellular pH (pH_{in}) measurement

To measure the pH_{in} and establish the calibration curve, two probes are required to cover the range of pH. CDCF was used for pH values ranging from 3 to 4.5 (Nedergaard et al., 1990), and cFDA SE was used for pH values ranging from 4 to 7 (Breeuwer et al., 1996; Breeuwer and Abee, 2000a).

Cells containing CDCF or CFDA SE were centrifuged (13,000 *g*, 90 s), and the pellet was resuspended in 3 mL of McIlvaine's buffer (or phosphate buffer) at the pH value equal to the pH of the culture (5 or 3.2) before FCM analysis. The fluorescence intensity of the cells was directly linked to the pH_{in} in the range of the calibration curves. Calibration curves were determined for each cell sample. After staining, cells were centrifuged (13,000 *g*, 90 s) and resuspended in McIlvaine's buffer with pH values ranging from 3 to 7. Five microlitres of nigericin (0.2 mM in ethanol) and 5 μL of valinomycin (0.2 mM in ethanol) were added to equilibrate the pH_{in} and the pH medium (pH_{ex}) before FCM analysis.

2.8. Membrane potential evaluation

One millilitre of culture was centrifuged (13,000 *g*, 1 min) and the pellet was resuspended in 1 mL of McIlvaine's buffer, pH 7.3, and incubated for 20 min at 40 °C in the presence of 5 μL of DiBAC (3) (0.5 $mg.mL^{-1}$ in DMSO). Samples stained with $DiBAC_4$ (3) were analysed by FCM. The result indicated the depolarised cell number and the depolarisation level: the higher the fluorescence intensity was, the more the cells were depolarised.

2.9. Flow cytometry analysis

FCM analysis was performed with a CyFlow SL cytometer (PARTEC, Sainte Geneviève des Bois, France). The cytometer was equipped with a solid blue laser emitting at 488 nm, and four band-pass filters: a forward-angle light scatter (FSC) combined with a diode collector, a side-angle light scatter (SSC) and two fluorescence signals collected with photomultiplier tubes, a 530-nm band-pass filter (515–545) to collect green fluorescence (FL1 channel), and a 630-nm long pass filter to collect the red fluorescence (FL2 channel). The FCM analysis was performed using logarithmic gains and specific detector settings adjusted on a sample of unstained cells in order to eliminate cellular autofluorescence. Gating on FSC/SSC was used to discriminate bacteria from the background.

Data were collected and analysed with FlowMax software (PARTEC, Sainte Geneviève des Bois, France). The subpopulations were identified using dot plots. Gates were defined in the dot plots of FSC, SSC, green fluorescence and red fluorescence, thus allowing the software to separate the different events. Data were analysed using statistical tables that indicate numbers and percentages of stained cells determined by each detector, as well as the fluorescence intensity of each fluorescent signal.

2.10. Microscopic analysis

Labelled fluorescent cells were analysed with an Axiostar + epifluorescence microscope (CARL ZEISS, Le Pecq, France) equipped with a 50-Watt mercury lamp. A JAI M70 camera (Imazys, Suresnes, France) linked to ITEX software (Imazys, Suresnes, France) was used to take microphotographs.

2.11. Malic acid concentration

The kinetics of malic acid consumption were monitored using the enzymatic method (Enzytec, Biopharm, Saint Dizier au Mont d'Or, France). One millilitre of the culture was centrifuged and the supernatant was used to determine the residual quantity of malic acid once daily.

2.12. Energy charge

The energy charge was calculated as the ratio as follows: EC = (ATP + 1/2 ADP)/(ATP + ADP + AMP).

For the measurement of nucleotide acids, ADP and AMP were converted to ATP according to (Gorman et al., 2003).

For the extraction of nucleotides, 800 μL of DMSO and 100 μL of 10 mM EDTA were added to 100 μL of cell suspension precisely adjusted to 10^6 $cells.mL^{-1}$, mixed for 1 min and then centrifuged (13,000 *g*, 5 min). Nine hundred microlitres of supernatant were added to 4 mL of 20 mM Tris buffer and stored at −20 °C before analysis. The ATP concentration was measured with an ATP meter (MicroLumat LB 96PEG&G Berthold, Germany).

2.13. Evaluation of activity of *O. oeni* culture

The aim of this experiment was to establish the link between the physiological cellular state of *O. oeni* cells (pH_{in}) and the activity of the cells measured by the lag phase of the MLF. In order to evaluate the role of the physiological cellular state of *O. Oeni* on the initiation of MLF, a culture of *O. oeni* in FT80 medium, pH 3.2, was performed, and pH_{in} and malic acid concentration were measured at different times. At each of these times, 10^8 *O. oeni* cells were taken from this culture and inoculated in 100 mL of fresh FT80 medium, pH 3.2, and incubated at 30 °C. Malic acid was analysed in these sub-cultures once a day throughout MLF. The MLF lag time was calculated as the time where the initial malic acid concentration remained constant. The duration of MLF was the sum of the MLF lag time and the malic acid consumption time (from 5 g/L to 0). The same experiment was carried out on white wine: samples were taken during MLF in wine, analysed (malic acid concentration and pH_{in}), and 1 mL (adjusted to 10^8 cells) was transferred to 100 mL of fresh white wine (malic acid concentration adjusted to 5 g/L), incubated at 20 °C. MLF was monitored by measuring the malic acid concentration once daily. The MLF lag time and the malic acid consumption time were calculated as described above. The shorter the MLF lag time was, higher the activity of *O. oeni* cells was.

3. Results

3.1. Growth curve, viability after BacLight staining and culturability assessment

Growth curves of *O. oeni* established by plate counts and FCM after BacLight™ staining are shown in Fig. 1. At pH 3.2, the duration of the growth was longer than at pH 5: the lag phase was 24 h, the exponential phase began as of 50 h, and the stationary phase after 168 h of culture. The final cell concentration was 2.28×10^7 $cells.mL^{-1}$. At pH 5, the lag phase was shorter (<24 h) and the stationary phase began as of 72 h. The final cell concentration was 6.3×10^7 $cells.mL^{-1}$. The FCM counts were well correlated with the plate counts throughout the culture for the two pH conditions. The R^2 correlation was 0.99 for the two culture conditions.

3.2. Transmembrane potential change during MLF

With the DiBAC probe, the fluorescence intensity increases with the depolarisation level of the cells. FCM was used to evaluate the change in the percentage of depolarised cells compared to total cells and the

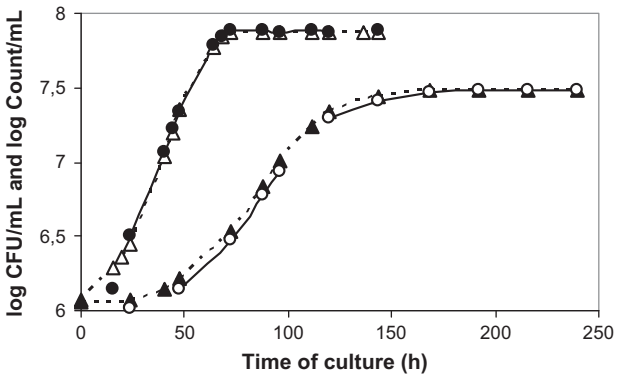

Fig. 1. *O. oeni* counts in FT80 broth at pH 5 (△): plate count; (•): FCM; pH 3.2 (▲) plate count, (○): FCM. The maximum standard deviations for these measurements were 0.073 for plate counts and 0.045 for FCM counts. The error tool bars were removed to make the graph clearer.

depolarisation level (fluorescence intensity of cells) (Fig. 2a). In FT80 medium at pH 3.2, the transmembrane potential of *O. oeni* cells was weak at the beginning and at the end of MLF, with 95% and 98% of depolarised cells and a high depolarisation level (FI = 50, arbitrary unit). During the exponential growth phase, the transmembrane potential of *O. oeni* cells increased since we observed only 7.5% of weakly depolarised cells at 168 h (strong decrease of fluorescent intensity level; FI = 18, arbitrary unit). Microscopic observations of *O. oeni* cells confirm the bright fluorescence of cells at the beginning of growth and at the end of the stationary growth phase and a weak fluorescence of cells in the exponential phase (Fig. 2b).

3.3. pHin change during MLF

The calibration curve is carried out at each measurement. The choice of one of the two probes is based on the value of each previous measurement. At the beginning of MLF in FT80 medium at pH 3.2, the pH_{in} of the cells was very low, increased during cell growth to a maximum of pH_{in} 6.1 at 168 h, which corresponded to the entry into the stationary phase, and then decreased to pH 3.26 at the end of the stationary phase. Surprisingly, the pH_{in} at the beginning and at the end of MLF seemed to be close to the medium pH. Measuring the change in pH_{in} during MLF in FT80 medium at pH 5 confirmed that the *O. oeni* cell pH_{in} was equilibrated with the medium pH at the beginning and at the end of MLF, regardless of the pH (Fig. 3).

Thus, in FT80 medium, pH 3.2, there was a change in pH_{in} of almost three pH units during the growth of *O. oeni*, which corresponded to a ΔpH ($pH_{in} - pH_{ex}$) ranging from zero at the beginning of the culture to 2.8 at the entry into the stationary growth phase. The pH_{ex} increased slightly, from 3.2 to 3.3 at the end of the exponential growth phase.

3.4. Vitality and energy charge changes during MLF

In FT80 medium, pH 3.2, the vitality measured by the percentage of cF extrusion from cells (descriptor ΔFI_{15}) by FCM was 28% at the

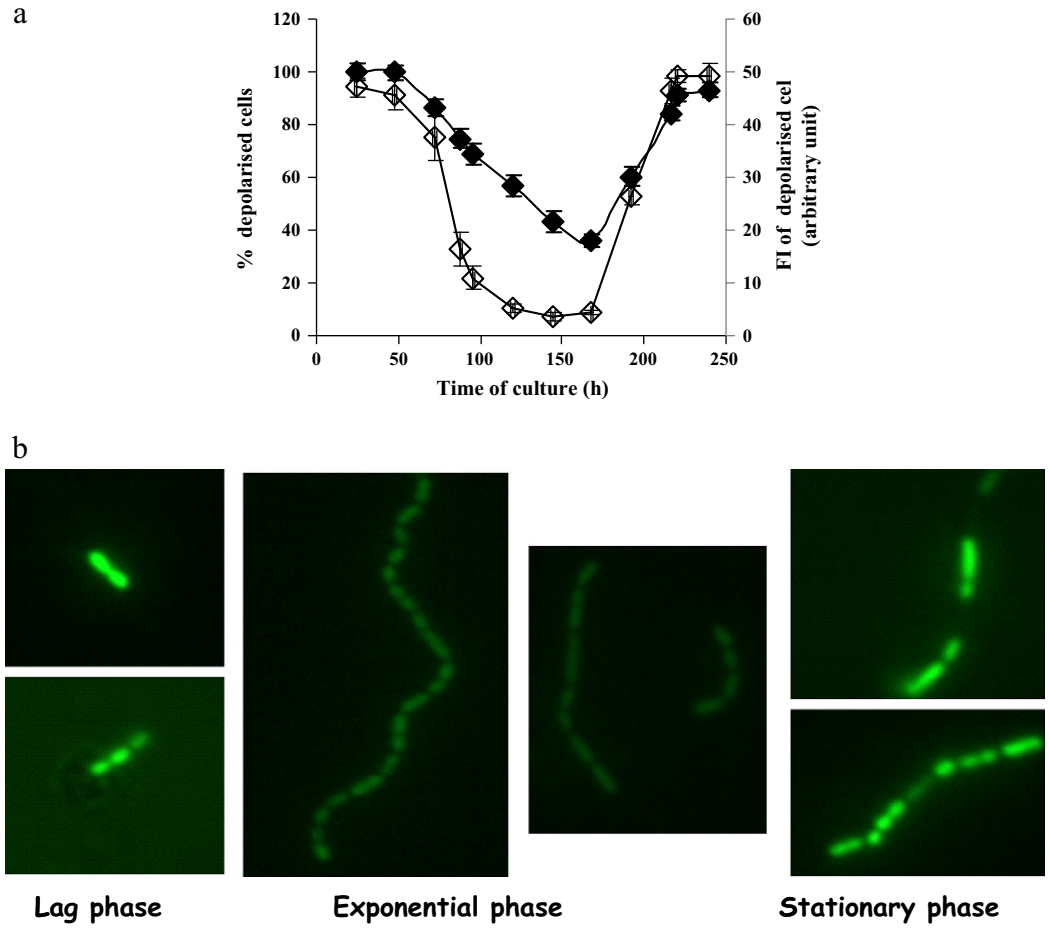

Fig. 2. Change in *O. oeni* cell depolarisation during MLF in FT80, pH 3.2. a: Percentage of depolarised cells (◇) and depolarisation level (FI) (♦) after DiBAC staining. The percentage of depolarised cells decreased up to 168 h (maximum polarisation). b: Photograph of *O. oeni* cells during MLF after DiBAC staining. The fluorescence intensity of cells after DiBAC staining was lower in the exponential phase than in the lag phase or at the end of the stationary phase.

beginning of the growth, increased to 82% at 168 h, and decreased to 30% at the end of the stationary growth phase. The maximum value of the energy charge also occurred at 168 h (Fig. 4). These measurements were performed without the addition of energetic substrate, corresponding to the intrinsic values of vitality and energy charge in the cells during MLF.

3.5. Malic acid consumption and cellular parameters of O. oeni changes during MLF

In Fig. 5a, we have superposed the consumption of malic acid and pH_{in} change during the growth of *O. oeni* in FT80 medium, pH 3.2. The malic acid concentration reached zero at 168 h and the pH_{in} increased from 3.2 to 6.1 during malic acid consumption. When the malic acid concentration tended to zero, the pH_{in} rapidly decreased. All these results were validated in wine and the data are shown in Fig. 5b: in wine, due to the necessity to adapt to ethanol concentration and to the temperature (20 °C instead of 30 °C in synthetic medium), the MLF lag phase was more time-consuming. However, like in synthetic medium, the polarisation level of cells, pH_{in} and vitality were weak during the lag phase of the MLF, increased during growth with the consumption of malic acid to reach a maximum when malic acid was exhausted, and immediately decreased to their initial values. The maximum pH_{in} value was the same in wine and in synthetic medium for an equal malic acid concentration (5 g/L).

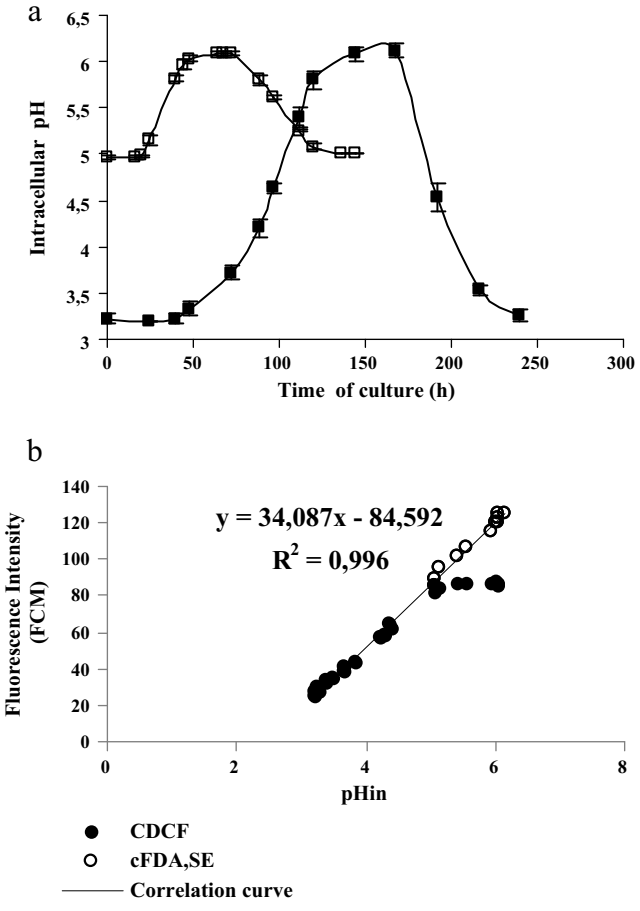

Fig. 3. a: Change in pH_{in} during MLF in FT80 medium, pH 3.2 (■), and FT80 medium, pH 5 (□). At the beginning and at the end of MLF, pH_{in} is equal to pH_{ex}. pH_{in} increased to 6.1 during growth. b: Calibration curve with CDCF and CFDA SE.

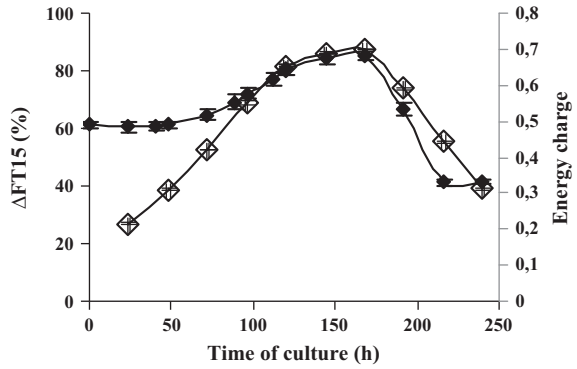

Fig. 4. Vitality (◊) and energy charge (•) during MLF in FT80 medium, pH 3.2. The two values increased up to 168 h of culture and then decreased.

3.6. Relationship between maximum pH_{in}, malic acid concentration and activity of O. oeni cells

FT80 medium pH 3.2, or aliquot of wine was inoculated with *O. oeni* cells from different stages of a pre-culture and then in various physiological states (various pH_{in}, polarisation levels and residual acid malic concentrations named "inoculum values" in Fig. 6). Regardless of the medium, synthetic or wine, the higher the pH_{in} and polarisation level (lower fluorescence intensity) of inoculated cells were, the shorter the lag phase of the MLF was (Fig. 6). On the contrary, the malic acid consumption time was the same, regardless of the physiological state of *O. oeni* cells before inoculation.

4. Discussion

4.1. Vitality descriptor

In this study, we propose a descriptor ΔFI_{15} to measure the vitality of cells. The principle of this measurement is based on the energy-dependent cF extrusion ability (Breeuwer et al., 1994). The cells were loaded with cF and the fluorescence intensity (FI_0) was measured. Then, during a second incubation, the cF was excreted even more rapidly when the cells had a high energy charge. The more active the cells were, the greater the extrusion was and, therefore, the greater the difference in the fluorescence of cells before and after incubation over the same time period was. This descriptor has been used for yeast (Bouix and Leveau, 2001) and *Lactobacillus* (Rault et al., 2008). cF extrusion was also used to assess the activity of *O. oeni* in several stress conditions with ethanol (Da Silveira and Abee, 2009). These latter authors added an energy source to the buffer and their measurements were performed at pH 5.5. In our descriptor, we compensated for the variations of pH_{in} during MLF on the measurement of fluorescence of the cF (pH-dependent) by taking the difference of fluorescence intensities ($FI_0 - FI_{15}$) divided by the initial fluorescence intensity (FI_0) into account. Otherwise, we did not use an energy source in our experiment to measure the extrusion of cF from the cells at each point in time in MLF conditions, and measurements were performed in pH 3.2 buffer (wine pH).

4.2. Cell parameter changes during MLF

The work of Augagneur et al. (2007) showed that the presence of citrate in buffer impacts the pH_{in} value by increasing the ΔpH. In preliminary experiments, we used two buffers (phosphate and citrate) for staining cells for pH_{in} measurements with the two probes, CDCF and CFDA SE. No significant difference was observed between the two buffers and a good correlation ($R^2 = 0.996$) was obtained between the two pH_{in} measurement series with the two buffers (data not

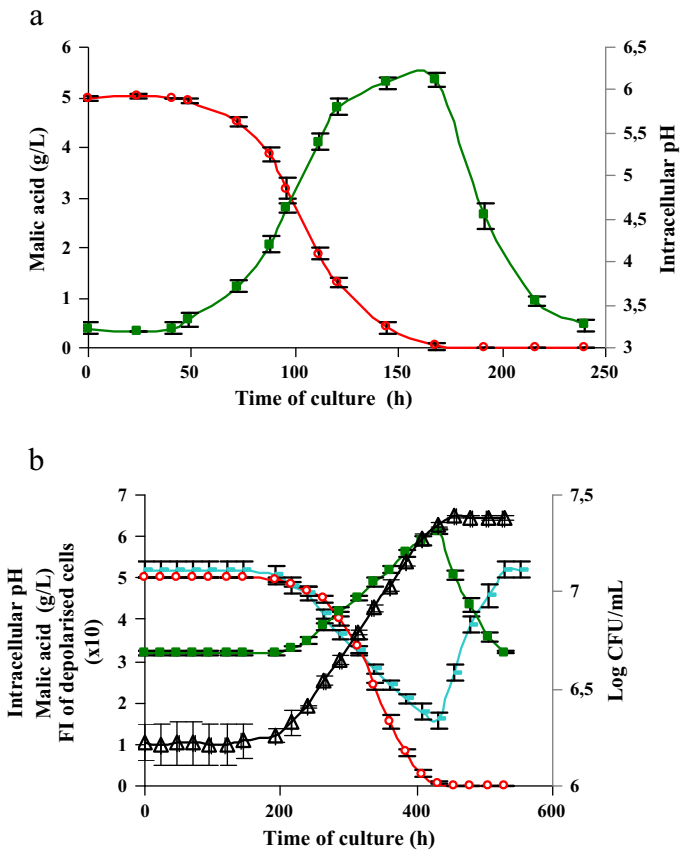

Fig. 5. Malate consumption and cellular parameters of *O. oeni* changes during MLF in FT80 medium, pH 3.2 (a) and in wine (b). a: FT80 medium, pH 3.2, malic acid (O), pH_{in} (■). The maximum pH_{in} increased until malic acid reached 0. b: White wine, malic acid (O), pH_{in} (■), FI depolarised cells (−) and plate count (Δ).

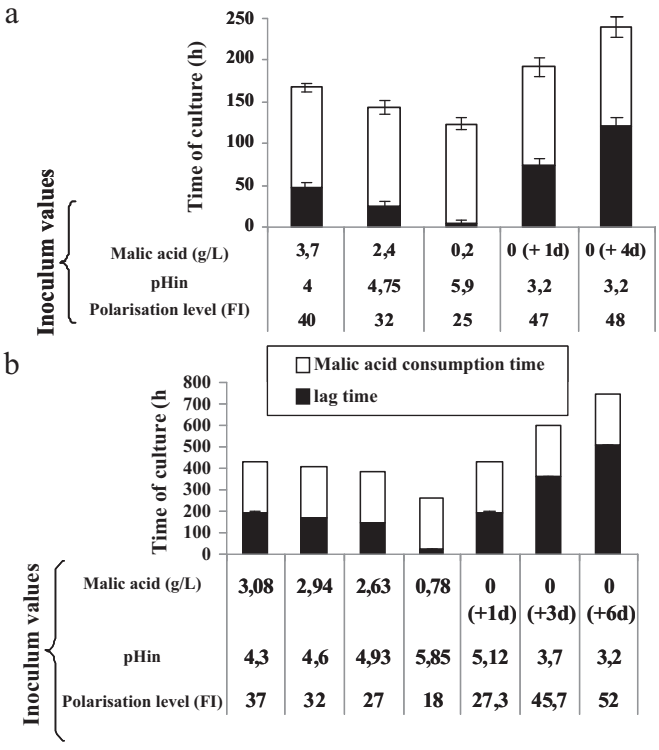

Fig. 6. Cellular parameters of *O. oeni* and lag time of MLF in FT80, pH 3.2 (a) and in white wine (b). The higher the pH_{in} and polarisation level (lower fluorescence intensity) of inoculated cells were, the shorter the lag time of the MLF was, but the malic acid consumption time remained constant.

shown). The difference with the results of Augagneur et al. (2007) can be explained by the fact that our MC1 strain is citrate-negative.

However, our results were in agreement with those of Augagneur et al. (2007) who observed a ΔpH equal to 2.6 (pH_{in} = 5.8) for a culture with a malic acid concentration of 37 mM, i.e., the same ΔpH as in our study with the same concentration of malate. On the contrary, the ΔpH was only equal to 1 (pH_{in} = 4.2) for the culture without malic acid; the ΔpH was thus greater when the culture under acidic conditions was performed in the presence of malic acid. These measurements were performed at only one point in time on the culture while in our work, we established the kinetic change of pH_{in} in the entire fermentation.

It is now well accepted that MLF is a proton motive force generating process (Cox and Henick-Kling, 1995; Loubiere et al., 1992; Salema et al., 1996b). Nevertheless, most of the previous studies were performed on cells harvested at the end of the exponential phase. Due to the simplicity and lower time consumption of FCM associated with fluorescent probes, we simultaneously measured pH_{in} and ΔΨ throughout MLF, both in synthetic medium and in wine, and determined their changes. Moreover, with the vitality descriptor ΔFI_{15}, we confirmed ATP synthesis, as suggested by (Galland et al., 2003). The kinetics of physiologic cellular changes of *O. oeni* are illustrated in Fig. 7: at the beginning of the growth, ΔΨ is low and pH_{in} is equal to pH_{ex} (ΔpH = 0). The vitality is weak. During the growth of *O. oeni*, ΔΨ increases (negative charge inside the cell) when malate enters the cell (Loubiere et al., 1992). According to Salema et al. (1996b), the malolactic enzyme clives malic acid, and lactic acid and CO_2 are co-excreted with protons, which causes the pH_{in} to increase to 6–6.1 (ΔpH = 2.8–2.9). This ΔpH allows the entry of protons and the increase of the proton motive force with the synthesis of ATP by the ATP synthase (Cox and Henick-Kling, 1989, 1995). When malic acid is totally exhausted, pH_{in}, ΔΨ and vitality decrease. These results were in accordance with the

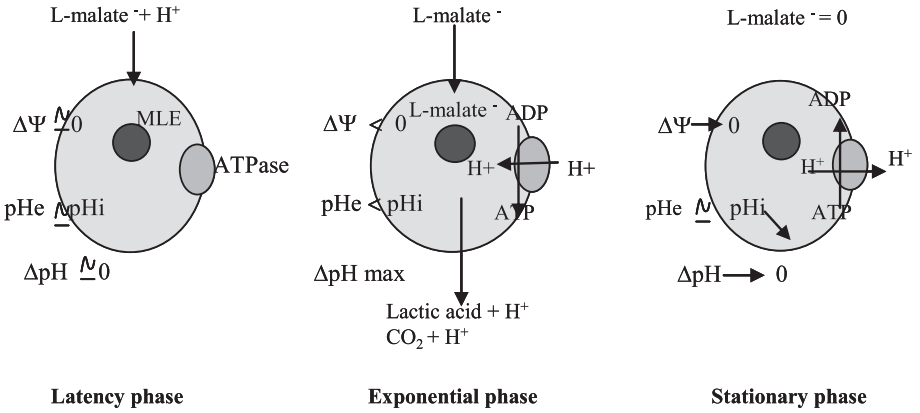

Fig. 7. Changes in force proton motive components and functioning of ATPase during MLF. At the beginning of the growth, ΔΨ was low and pH_{in} was equal to pH_{ex} (ΔpH 0). During the growth of *O. oeni*, ΔΨ increases when malate enters the cells, the malolactic enzyme clives malic acid, and lactic acid and CO_2 are co-excreted with protons, which causes the pH_{in} to increase. This ΔpH allows the entry of protons and the increase of the proton motive force with the synthesis of ATP by ATP synthase. When malic acid is totally exhausted, pH_{in} and ΔΨ decrease.

literature cited above. However, in the case of FCM, we specified the course of the cellular phenomenon during MLF from the kinetic point of view.

No values have been reported in literature for pH_{in} in cells not undergoing growth. Our measurements indicated that the pH_{in} value was equal to the pH of the medium ($pH_{in} = pH_{ex}$). This can be explained by the metabolism of the bacteria as described above. Nevertheless, we can wonder about the survivability of the bacterium under such harsh conditions.

4.3. Relationship between cellular parameters and activity of O. oeni

On the basis of these kinetics, we confirmed our hypothesis that the higher the vitality, the pH_{in} and the polarisation of inoculated cells are, the shorter the lag time of the MLF will be. In our experiments, in FT80 medium, the lag time varied from 4 h to 120 h, depending on whether the pH_{in} of inoculated *O. oeni* cells was equal to 5.9 or 3.2. In white wine, with the same range of pH_{in} of inoculated cells, the lag time varied from 24 h to 504 h (21 days), respectively. The lag phase was longer in wine than in synthetic medium, probably due to the presence of ethanol and other inhibitory compounds. However, the lag phase in wine was not longer when pre-cultured was performed in FT80 than in wine, i.e., 24 h from FT80 to wine or from wine to wine (data not shown), which suggests that the physiological state of cells was more important in reducing the lag phase than adaptation to ethanol. It is interesting to point out that the lag time rapidly increases in fresh medium after the malic acid concentration in pre-culture reaches zero. In practice, when inoculating wines from a pre-culture, the simple measurement of malic acid concentration could be used to control the physiological state of inoculation cells. Validation in red wine is in progress.

Use of freeze-dried starter cultures in MLF, either with direct inoculation or with re-hydrated cells, still provides random results. We think MLF is sometimes sluggish because there is no criterion to control the physiological state of cells at the time of inoculation. Given our results, we think it would be interesting to integrate these physiological cellular state parameters into the production of active functional freeze-dried starters or into the re-hydration phase to improve the onset of MLF.

5. Conclusion

FCM was quickly able to assess the pH_{in} and membrane potential of *O. Oeni* cells during MLF. The results obtained in synthetic medium at two pH values and in white wine showed that the pH_{in} underwent considerable changes during MLF. These variations are undoubtedly linked to the complex energy metabolism of *O. oeni*.

The simple measurement of the cellular parameters (pH_{in}, ΔΨ and ΔFI_{15}) makes it possible to better understand the cellular mechanisms of *O. oeni*. Together with malic acid concentration, it provides us with simple tools to evaluate cellular activity before inoculating bacteria in wine and to more effectively control malolactic fermentation.

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